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DEVELOPMENTAL BIOLOGY 177, 332-346 (1996)
ARTICLE NO. 0167

Induction of Embryonic Vasculogenesis by bFGF and LIF *in Vitro* and *in Vivo*

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The *de novo* formation of blood vessels (vasculogenesis) is an integral part of embryogenesis. Elucidation of the role of cytokine cooperation in vasculogenesis may lead to a better understanding of organogenesis, blood vessel regulation during tumorigenesis, and tissue injury. We have used embryonic stem cells to derive an endothelial cell line, designated IEM, which expresses a range of endothelial markers, including Von Willibrand Factor VIII related antigen, vascular cell adhesion molecule, platelet-endothelial cell adhesion molecule (CD31), and receptors for acetylated low-density lipoprotein. More importantly, IEM cells can be induced upon exposure to combinations of basic fibroblast growth factor and leukemia inhibitory factor (LIF) to proliferate and undergo vasculogenesis *in vitro*, resulting in the formation of vascular tubes and microcapillary anastomoses. Moreover, exposure to both cytokines conditionally permits IEM cells to specifically chimerize microvascular endothelium *in vivo* following blastocyst injection. These results indicate that bFGF and LIF together contribute to the induction and support of embryonic vasculogenesis in an isolated endothelial cell line. Our results provide evidence that combined actions of bFGF/LIF may play a role in mechanisms controlling blood vessel development.

INTRODUCTION

Blood vessels arise through the combined processes of vasculogenesis (*de novo* formation of vessels by differentiation of angioblasts) and angiogenesis (elongation of preexisting capillary heads). To date, vasculogenesis has been demonstrated only in the developing embryo, while angiogenesis mediates embryonic vessel formation, wound healing, and tumor vascularization (Folkman, 1995). The endothelial lineage arises from the primitive mesoderm during early pregastrulation development and depends upon endodermal induction (Palis *et al.*, 1995). Primitive blood islands coalesce in the yolk sac to form a capillary network at the same time that vasculogenesis occurs inside the embryo, where primitive endothelial cells, or angioblasts, differentiate *in situ* into vessel-forming structures (Parnadaud *et al.*, 1987). This intraembryonic vasculogenesis is characterized by the expression of the endothelial markers Von Willibrand factor VIII (vWFVIII), platelet endothelial cell adhesion molecule (PECAM-1), and *flk-1*, one of the tyrosine kinase receptors (RTK) for vascular endothelial growth factor (VEGF) (Coffin *et al.*, 1991; Baldwin *et al.*, 1994; Yamaguchi *et al.*, 1993). Interestingly, angioblasts, giving rise to the developing vasculature, are phenotypically similar to precursors which form vasoformative neoplasms. Indeed, the migratory and invasive nature of angioblast cells has been demonstrated experimentally in chick-quail chimeras in which migrating angioblasts can be observed actively moving throughout and around the graft site (Christ *et al.*, 1991). In virally induced hemangiomas, endothelioma cells stimulate proliferation of host endothelial cells after invading and penetrating existing vessels (Wilms *et al.*, 1991; Williams *et al.*, 1989). Regulatory pathways controlling normal embryonic angioblasts from uncontrolled invasion, random migration, and continued growth after completion of the homing process have yet to be described. Although the turnover time of adult endothelial cells is extremely long, mature endothelial cells are not permanently arrested and can be switched back to a proliferative state upon stimulation during wound healing or vascularization of tumors (Folkman and Shing, 1992). Endothelial cell lines retaining responsiveness to these switching mechanisms, particularly

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0012-1606/96 $18.00

in response to specific cytokines, would be very useful in fully understanding the molecular events underlying these functional changes in endothelial differentiation.

A number of angiogenic factors have been identified, such as acidic and basic fibroblast growth factor (bFGF), angiogenin, transforming growth factors α and β (TGF-α and TGF-β), tumor necrosis factor α (TNF-α), prostaglandins, and VEGF, as well as physiological inhibitors of angiogenesis (reviewed in Folkman and Klagsbrun, 1987; Folkman and Shing, 1992; Dickson *et al.*, 1995; Pandey *et al.*, 1995). However, there is not yet a complete understanding of the cytokines and signals controlling the initial emergence of angioblasts from mesoderm, induction of endothelium by endoderm, or the eventual organization and proliferation of the more differentiated endothelium into the precursors of the vascular tree. Expression of VEGF, the ligand for the two closely homologous RTKs *flk-1* and *flt-1*, has recently been shown to be associated with areas of the embryo undergoing vasculogenesis and can lead to aberrant vasculogenesis when overexpressed in mice (Millauer *et al.*, 1993; Drake and Little, 1995). Targeted inactivation of *flk-1* results in a phenotype completely lacking both vascular and blood island progenitor cells (Shalaby *et al.*, 1995). Through gene targeting, *flt-1*, in contrast to *flk-1*, has been demonstrated to be involved in the structural assembly of the vasculature, but not for the initial differentiation of angioblast cells (Fong *et al.*, 1995). Targeted inactivation of the TGF-β gene results in defects in both vasculogenesis and hematopoiesis, suggesting that TGF-β may play an important role in the development of the bipotential hemangioblast (Dickson *et al.*, 1995). Other groups have demonstrated the requirement of bFGF for the induction of vasculogenesis in embryoid bodies of quail (Krah *et al.*, 1994). Since vasculogenesis occurs spontaneously in mouse embryoid bodies, it is not yet known whether bFGF is absolutely required for vasculogenesis in mammalian systems (Risau *et al.*, 1988; Wang *et al.*, 1992). However, endodermally derived bFGF stimulates vitilline vasculogenesis through its receptors expressed in the developing yolk sac (Yasuda *et al.*, 1992). Furthermore, bFGF stimulates the expression of *flk-1* mRNA at early stages in quail blastodisc cultures and this expression has been hypothesized to be related to the differentiation of hemangioblasts from early epiblasts (Flamme *et al.*, 1995). The RTKs *tek*, *Tie-1*, and *Tie-2* all have recently been shown to be involved in the normal development of the embryonic endothelial lineage, as expression patterns of these genes indicate specific localization in developing endothelium shortly after *flk-1* expression is detected (Millauer *et al.*, 1993; Yamaguchi *et al.*, 1993; Sato *et al.*, 1993). Null mutations of *Tie-1* and *Tie-2* RTKs reveal that expression of these genes is important for vasculogenesis and angiogenesis. Furthermore, null mutants for the RTK *tek* show defects in vascular and heart development (Dumont *et al.*, 1994; Sato *et al.*, 1995). However, studies of the signaling responses of these RTK molecules expressed later in endothelial development must await the characterization of their respective ligands.

The importance of interaction and cooperation of angiogenic factors in the regulation of endothelial growth and differentiation during the development of the mammalian vasculature is not well understood. In other previously characterized developmental systems, cytokine interactions have been observed to cause profound differentiative influences on early progenitor cell subsets, with differentiation proceeding either forward, to a more mature cell type (Palacios *et al.*, 1995), or backward, to a more primitive cell type (Pittack *et al.*, 1991; Resnick *et al.*, 1992; Matsui *et al.*, 1992). Embryonic stem (ES) cells will differentiate in a hematopoietic lineage-specific manner when cultured in combinations of interleukin-3 (IL-3), leukemia inhibitory factor (LIF), cytokine-family member interleukin-6 (IL-6), and liver stromal cell supernatant in the presence of feeder cell layers (Palacios *et al.*, 1995). Primordial germ cells will proliferate and transdifferentiate into primitive pluripotential embryonic stem cells *in vitro* when cultured in combinations of stem cell factor (SCF), bFGF, and LIF (Resnick *et al.*, 1992; Matsui *et al.*, 1992). *In vivo*, the LIF-cytokine family member ciliary neurotrophic factor (CNTF) can interact with bFGF to effectively maintain a primitive differentiative stage of developing skeletal muscle fibers (English and Schwartz, 1995). Such cytokine interactions with LIF or LIF family members in a variety of other early embryonic differentiation systems begs the question of whether LIF might cooperatively interact with angiogenic cytokines such as bFGF in signaling mechanisms regulating embryonic endothelial cells. Although little is known about the role of LIF family cytokines in vasculogenesis, recent observations indicate that this cytokine may indeed play a role in early endothelial development and angiogenesis. Transplantation experiments in chick have shown that LIF influences the ability of cardiac projenitors to rescue heart development (Kirby *et al.*, 1993). Targeted inactivation of the low-affinity LIF receptor results in a reduction of the fetal blood vessel component in the developing placenta, suggesting that formation of the fetal vasculature may depend partly on LIF (Ware *et al.*, 1995). LIF appears to exert differing actions on endothelial growth depending upon the tissue origin of the endothelial cells studied, stimulating growth in adrenal cortex capillary endothelial cells while inhibiting growth of aortic endothelial cells (Ferrara *et al.*, 1992). Finally, LIF may be associated with endothelial pathological processes as abnormally elevated levels of LIF have been detected in patients with giant cell arteritis, a degenerative disease of the blood vessel wall (Lecron *et al.*, 1993). No studies to date address the combined effects of bFGF and LIF upon angiogenesis or vasculogenesis.

Model systems retaining responsiveness to angiogenic and vasculogenic signals are needed to fully understand the key regulators directing blood vessel development, progression, and assembly. These regulatory molecules could be relevant to both developmental and tumor processes. We hypothesized that isolated embryonic endothelium would retain the ability to recapitulate vasculogenesis after immortalization *in vitro*. Our central objective was to derive a homogeneous model system in which specific cytokines could be used to induce proliferative and differentiative

changes in isolated embryonic endothelial cells. Here we describe such a system in which the combined actions of bFGF and LIF induce vasculogenesis in an embryonic endothelial cell line both *in vitro* and *in vivo*. Our results indicate that the combined action of these cytokines influences the developmental pathway traversed by embryonic endothelial cells and suggest that these cytokines may play a role in directing the early formation of the vascular tree.

MATERIALS AND METHODS

Derivation of the IEM Cell Line

ES cells, which are known to give rise to vasculogenic progenitors *in vitro* (Risau *et al.*, 1988), were grown in the presence of LIF on gelatinized tissue culture plates in the absence of STO feeder cells. The ES cells were an early passage of the CCE line (Bradley *et al.*, 1984). Confluent plates of ES cells were trypsinized and replated in complete Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (FBS), LIF (1000 units per ml), and testis conditioned medium (TCM) (1:100 dilution of a 0.45 μm-filtered solution made by homogenizing one adult mouse testis per milliliter PBS). The TCM served as an enriched source of endogenous cytokines and solubilized exracellular matrix proteins and has a profound effect on ES cell differentiation (unpublished observations). After 18 hr, the culture medium was replaced with sterile-filtered medium conditioned by $\psi 2$ cells producing the pZIPTEX retroviral vector containing the coding sequences for the early region of the SV40 Large T antigen and the dominant selectable neomycin resistance gene (Brown *et al.*, 1986). The viral titers of the packaging cell supernatants ranged from 10^6 to 10^8 neopositive colony-forming units per milliliter when tested on NIH 3T3 fibroblasts. The $\psi 2$ supernatant was supplemented with LIF and TCM at the concentrations specified above. After infection, the cultures were incubated with selection medium containing geneticin (G418; Gibco) at 0.5 mg/ml, LIF, and TCM. Colonies surviving G418 selection were picked with glass cloning cylinders, expanded, and maintained in medium supplemented with G418 at 0.25 mg/ml. The cloned IEM line was cultured in the presence of LIF and TCM for approximately 1 month, after which time the line was switched to complete DME containing 10% FBS and 0.25 mg/ml G418. For all experimental procedures, IEM cells were cultured in the absence of G418 for 1 week prior to use.

Endothelial Phenotyping of IEM Cells in Vitro

Immunocytochemistry for endothelial specific markers was performed on acetone-fixed coverslip cultures of IEM. Staining for vWFVIII (Wagner *et al.*, 1982) was performed using a rabbit polyclonal anti-vWFVIII-related antigen (Dako) with a peroxidase-conjugated goat anti-rabbit second step (Promega). Staining for vascular cell adhesion molecule (VCAM-1) and PECAM-1 (CD31) (Osborn *et al.*, 1989; Baldwin *et al.*, 1994) was accomplished using rat monoclonal anti-mouse-VCAM-1 and anti-mouse-PECAM-1 antibodies (Pharmingen) and an alkaline phosphatase-conjugated goat anti-rat second step (Promega). All reactions involving alkaline phosphatase reagents were developed in the presence of levamisole to inhibit endogenous tissue alkaline phosphatase reactions. All staining was carried out at room temperature in 0.05 *M* Tris-buffered saline, pH 7.5, in a humidified chamber. Acetylated low-density lipoprotein (a-LDL) uptake (Voyta *et al.*, 1984) was determined by incubating coverslip cultures of IEM cells or nonendothelial fibroblast control cells with 10 μg/ml of dil-coupled a-LDL (Molecular Probes) for 4 hr in medium containing 10% FBS under normal tissue culture conditions. After incubation, the coverslips were retrieved, washed twice in PBS, mounted with glycerol-gelatin, and photographed under a fluorescence microscope using a rhodamine filter.

Cytokine Reprogramming of IEM Cells to Vasculogenic Colonies

The IEM cells were plated at 3.5×10^5 cells/ml in tissue culture dishes in medium containing 10% FBS and allowed to adhere for 3-4 hr. This medium was then replaced with medium containing 1% serum, LIF (1000-1500 units/ml), and bFGF (5-10 ng/ml). Cultures were refreshed with bFGF and LIF on a daily basis. Control cultures consisted of IEM cells plated and maintained in the same manner but without cytokines. For the induction of vessel-like structures *in vitro*, IEM colonies induced by bFGF/LIF exposure for 3 days were picked out and transferred to 35-mm plates containing a layer of 1 ml of Matrigel basement membrane matrix (Collaborative Biomedicals) covered with DMEM containing 10% FBS. Untreated IEM cells were transferred to separate Matrigel-coated plates for negative controls. Matrigel cultures were photographed after 2 days.

Matrigel-embedded vasculogenic colonies were also fixed in Bouin's fixative, dehydrated, and embedded in glycomethacrylate plastic embedding compound. Hardened blocks were then thin sectioned at 2 μm thickness, stained with toluidine blue, and mounted in permanent mounting medium for viewing the internal architecture of vasculogenic colonies.

Growth Curves

Growth stimulation of IEM by bFGF (Gibco) and LIF (ESGRO; Gibco) was determined using the sulforhodamine B (SRB) assay, as previously described (Skehan *et al.*, 1990). Briefly, cells were plated at 4000 or 7500 cells per well in 96-well plates (Costar) in medium containing 10% serum and allowed to adhere for 4 hr. The medium was then replaced with medium containing 1% serum in the presence or absence of varying concentrations of βFGF and LIF. Twenty-four or 72 hr later, the monolayers resulting from platings of 4000 and 7500 cells per well, respectively, were fixed with 16.7% trichloroacetic acid. The wells were then rinsed and stained with 0.4% SRB in 1% acetic acid. After a further washing in 1% acetic acid and drying, the SRB was solubilized with 10 m*M* Tris base, and the absorbances were read at 530 nm in a Titertek multiwell plate reader.

Blastocyst Injection of IEM Cells and Analysis of Chimeras

Approximately 25-30 IEM cells from pools of individual colonies generated by culture in 5-10 ng/ml bFGF and 1500 U/ml LIF were picked with a drawn-out Pasteur pipette and injected into C57/B6 host blastocysts at the 3.5 days postcoitum stage. Control injections were done using the same number of untreated IEM cells cultured in the absence of either cytokines or G418. The injected blastocysts were implanted into the uteri of pseudopregnant B6CBAF/J foster mothers. After weaning, the pups (and in some cases the adult offspring) were screened for the presence of the simin virus 40 (SV40) Large T genome by PCR and Southern blot analysis of genomic DNA. The initial screening for chimerization

was accomplished by PCR amplification of SV40 Large T sequences in DNA isolated from the tails of offspring resulting from blastocyst injections. The SV40 Large T primers used were: 5′ TCCAACCTATGGAACTGATG 3′ (sense) and 5′ AGTCAAGGCACTATACATCA 3′ (antisense). Reactions were carried out in a volume of 40 μl for 40 cycles. The amplicon resulting from the PCRs of IEM and mouse tissue DNA was verified to be specific by probing Southern blots of the PCR products using radiolabeled SV40 Large T sequences spanning the putative amplicon as a hybridization probe. For Southern blots of genomic DNA, 20 μg of DNA was digested with *BclI*, electrophoresed on a 1% agarose gel, and transferred to nylon membranes under denaturing conditions. The blots were then cross-linked with UV light (Stratalinker) and probed with a ^{32}P-labeled (Feinberg and Vogelstein, 1983) 1500-bp fragment of the SV40 early region situated 3′ of the single *BclI* restriction site present in the retroviral vector (Brown *et al.*, 1986).

Immunocytochemistry was performed on acetone-fixed frozen sections of IEM chimeric tissues to localize IEM cells using the SV40 Large T antigen as marker. The Large T epitope was stained using a biotinylated anti-Large T mouse monoclonal antibody (Pharmingen). Acetone-fixed frozen sections were preblocked with a biotin–avidin blocking kit (Vector Laboratories). After incubation with anti-Large T, the staining reaction was developed with either an avidin–peroxidase or an avidin–alkaline phosphatase chromogen substrate (Sigma). Chimeric tissues incubated with an irrelevant biotinylated mouse anti-rabbit IgG were used as negative controls. For double labeling studies, sections were first stained for Large T as described above and then stained with rabbit anti-vWFVIII using FITC-conjugated goat anti-rabbit second antibody. Double labeled slides were duplicate photographed under high dry 400X or oil immersion under light and fluorescent (fluorescein filter) conditions.

Animals

All experiments involving animals were performed in accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals at Children's Hospital Research Foundation in Cincinnati, Children's Hospital Boston and Dana Farber Cancer Institute. Animal experiments were approved by the Animal Care Committees at these institutions.

RESULTS

Derivation of an Embryonic Endothelial Cell Line from ES Cells

Embryonic stem cells can differentiate into primitive mesenchyme containing vasculogenic progenitors *in vitro* (Risau *et al.*, 1988). Therefore, to isolate representatives of early vasculogenic cells, a retroviral vector containing the SV40 Large T antigen was used to selectively immortalize differentiated ES cell derivatives. One of the resulting clones showed a mesenchymal phenotype as demonstrated by the expression of vimentin mRNA but not cytokeratin mRNA (not shown). This clone was expanded and established as a permanent cell line. Southern and Western blot analyses verified that the cells had integrated the SV40 genome and expressed Large T antigen (see below).

The karyotype of the cell line was determined to be XY, with a mean chromosome number of 54. The cell line expresses endothelial markers such as vWFVIII, VCAM-1, and PECAM-1 (CD31), and internalizes aLDL (Figs. 1a–1d, respectively). The heterogeneity in staining patterns for VCAM-1 and PECAM-1 in the IEM cells (Figs. 1b and 1c) was also observed in human umbilical vein endothelial cell (HUVEC) positive control preparations (not shown) and has been reported previously by other groups as a characteristic of marker expression by cultured endothelial cells (Masek and Sweetenham, 1994). These marker expression characteristics clearly define the cell line as having an endothelial phenotype (Wagner *et al.*, 1982; Osborn *et al.*, 1989; Bevilacqua *et al.*, 1987; Kwee *et al.*, 1995; Coffin *et al.*, 1991; Baldwin *et al.*, 1994; Voyta *et al.*, 1984; Alles and Bosslet, 1988; Vecchi *et al.*, 1994). The endothelial mouse cell line was designated IEM (Immortalized-(embryonal)-endothelium-mouse).

bFGF and LIF Can Reprogram IEM Cells to Form Vasculogenic Colonies Containing Primitive Blood Vessels in Vitro

Exposure of the IEM cells to a combination of bFGF and LIF results in a change in morphology characterized by a shift from monolayer to colony phenotype in 3 days (Fig. 2b). The IEM cells do not form colonies in the presence of LIF alone (Fig. 2c). Although the IEM cells display some colony formation in the presence of bFGF alone (Fig. 2d), the maintenance and continued growth of the colonies depends upon the simultaneous presence of LIF. Such colonies never form in the absence of cytokines (Fig. 2a) and removal of bFGF and LIF results in the reversion of the IEM colonies to a flat morphology over 2–3 days.

The IEM cells show increased growth in response to bFGF alone, LIF alone, and combinations of both cytokines (Figs. 3a–3c, respectively). The growth response of IEM cells was linear over at least a 3-day period. The maximal growth response of IEM was achieved using a concentration of 5–10 ng/ml of bFGF, while optimal concentrations of LIF ranged between 250 and 2000 U/ml. In both cases, higher concentrations of either cytokine decreased the level of growth stimulation (Figs. 3a and 3b). Combinations of both cytokines produced a partially additive growth response on IEM cells (Fig. 3c). IEM growth responses were optimal when bFGF concentrations between 5 and 10 ng/ml and LIF concentrations between 250 and 2000 U/ml were used (Fig. 3c). Thus, the optimal concentration of each cytokine for a growth response on IEM cells is not affected by the presence of the other. These results demonstrate that in addition to a differentiative signal to shift phenotype, a proliferative signal is simultaneously induced by bFGF/LIF exposure in IEM cells.

Matrigel was used in order to provide a collagen-containing matrix for further support of differentiation of cytokine-induced IEM colonies (Zimrin *et al.*, 1995). When plated onto Matrigel basement membrane matrix, the IEM colonies resulting from cytokine exposure sprout complex blood vessel structures within 2–3 days, characteristic of

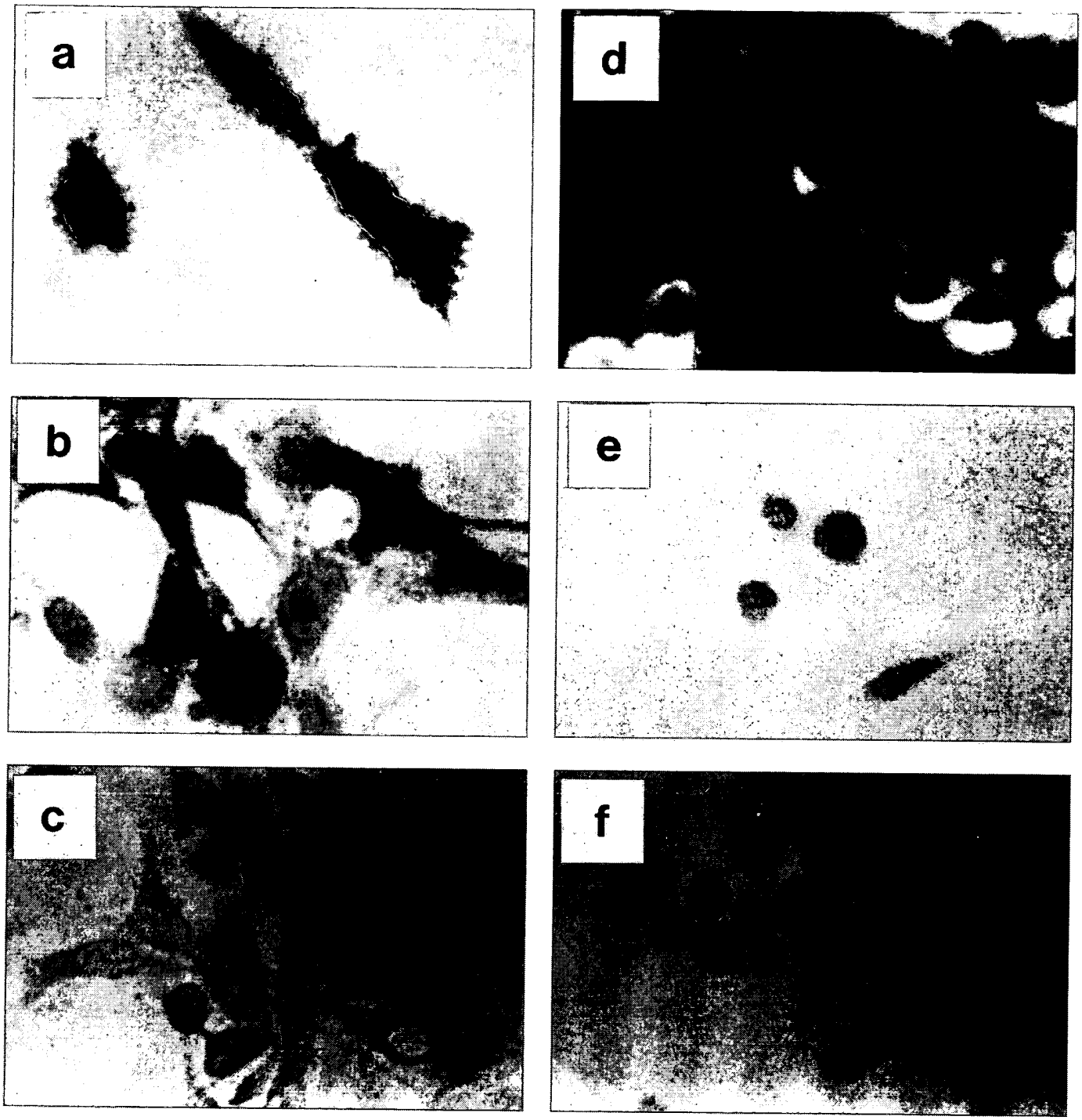


FIG. 1. Endothelial-specific markers expressed in the IEM cell line *in vitro*. (a) Immunoperoxidase staining with a rabbit anti-vWFVIII-related antigen. (b) Immunoalkaline phosphatase staining with rat anti-vCAM-1. (c) Immunoalkaline phosphatase staining with rat anti-PECAM-1 (CD31). (d) Fluorescence staining of IEM cells after specific uptake of DiI-labeled acetylated-LDL; control fibroblast cells were negative (not shown). (e) Absence of staining with nonimmune rabbit serum (NRS). (f) Absence of staining with rat IgG isotype control.

vasculogenesis (Figs. 4a–4c). Consistent with developing embryonic blood vessels, thin sectioning reveals that the IEM-derived blood vessel structures contain lumens and are predominantly composed of walls as thick as a single endothelial cell (Figs. 4d and 4e). In addition, capillary anastomoses are found in these colonies (Fig. 4f). Most likely due to the low concentrations of endogenous bFGF present in Matrigel (Collaborative Biomedical product specifications),

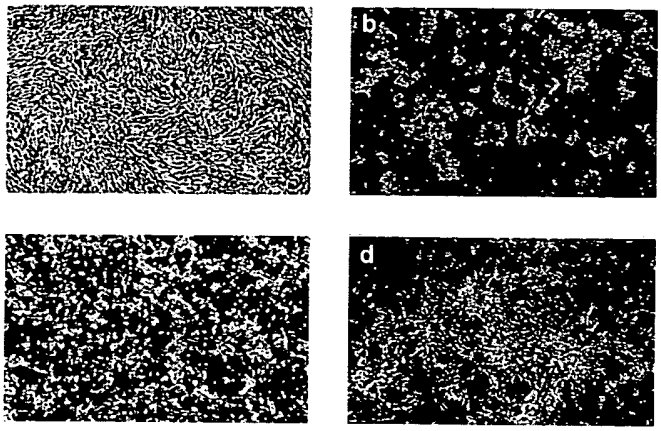


FIG. 2. bFGF/LIF exposure induces phenotypic conversion of IEM monolayers to vasculogenic colonies. (a) Control IEM cells (no cytokines) on Day 3 of culture; phase-contrast. (b) Day 3 culture of IEM cells in the presence of bFGF and LIF showing enlarging colonies; phase-contrast, 20X. (c) Day 3 culture of IEM cells in the presence of LIF alone; phase-contrast, 20X. (d) Day 3 culture of IEM cells in the presence of bFGF alone; phase-contrast, 20X.

sprouting structures could sometimes be observed after untreated IEM cells were transferred to Matrigel. However, tube formation in untreated cultures is delayed for up to 2 days and is significantly less extensive when compared to cultures exposed to cytokines. Both a separate ES-derived SV40 Large T antigen immortalized cell line and STO embryo fibroblasts fail to undergo any observable phenotypic shift after culture in bFGF and LIF, nor do they form vessel-like structures on Matrigel. Interestingly, primary cultures of HUVEC cells also did not show a shift to colony phenotype upon exposure to the same concentrations of bFGF and LIF (not shown). These findings indicate that bFGF/LIF exposure induces a specific phenotypic shift of the IEM cells leading to the onset of vasculogenic changes *in vitro*. This result suggests that embryonic vasculogenesis may be supported by combined actions of bFGF and LIF *in vivo*.

Exposure to bFGF and LIF Permits IEM Cells to Chimerize Developing Blood Vessels in Vivo

To examine the possibility that bFGF/LIF programming influences the potential of IEM cells to contribute to developing blood vessels in an *in vivo* setting, mouse blastocysts were injected with either cytokine-exposed (bFGF and LIF) or untreated IEM cells. Only the cytokine-exposed IEM cells were able to contribute to vascular chimeras in resulting mice (Fig. 5, panels 1 and 2; Table 1). As expected, no chimerism could be detected by coat color. However, PCR amplification revealed that tail DNA from 29% of the animals derived from injections of cytokine-exposed IEM cells contained SV40 Large T antigen sequences (Table 1). The amplicons observed after PCR of tail DNA were confirmed to be specific for SV40 Large T antigen by Southern blot analysis using a Large T specific cDNA probe (Fig. 5, panel 1, a). PCR followed by Southern analysis of tail DNA from all offspring injected with untreated IEM cells showed no SV40 Large T sequences (not shown). Moreover, PCR/Southern blot analysis of organs in which the level of chimerism obtained with cytokine-exposed IEM cells was more abundant (spleen and thymus) failed to reveal chimerism in animals resulting from blastocyst injection of untreated IEM cells. No SV40 sequences were detected in these tissues, indicating that cytokine exposure was necessary for the ability of IEM cells to chimerize vascular tissues (not shown). The *BclI* restriction site, which is present in the pZIP-TEX SV40 sequences but absent in the LTR, was utilized to verify concordant proviral integration sites in the IEM cells and chimeric mouse genomes. Southern blots of *BclI*-digested DNA probed with a Large T fragment representing sequences upstream of the restriction site showed a similar banding pattern in both tissues of the tail-positive mice and in the IEM cells, demonstrating that the tissues were chimeric rather than positive due to infection during development (Fig. 5, panel 1, b). Similar banding patterns between IEM cells and chimeric tissue was also observed using *pstI* enzyme digests [which also cuts once in the Large T gene and not in other viral sequences (not shown)]. The slight differences observed in the Southern blot banding intensity between IEM and tissue DNA are most likely due to the extreme differences in sample homogeneity, with IEM being pure where experimental tissue is a mix of IEM and nonchimeric tissues. A separate ES-derived SV40 Large T antigen immortalized cell line (designated ESD-2) which was isolated simultaneously with IEM using the pZIP-TEX vector, showed a *BclI* banding pattern on Southern blotting that was completely different from that found in the IEM cells and chimeric tissues, demonstrating that the banding pattern in IEM was unique to the IEM cell line (Fig. 5, panel 1, c). Chimerism was detected by Southern blot in spleen, thymus, muscle, and brain while levels in lung, liver, heart, and uterus were below the level of detection (for example, compare heart and thymus lanes in Fig. 5, panel 1, b).

Immunocytochemistry on frozen sections of tissues from chimeric animals indicated that the location of Large-T-positive IEM-derived cells was consistent with the vascular compartment. Figure 5 (panel 2, a–f) demonstrates the localization of Large T positive cells in the splenic red pulp sinusoids and in the microcapillaries of the brain. The Large-T-positive cells were found within areas of vWFVIII staining in consecutive sections of the same organs (red pulp surrounding trebeculae in spleen and microvessel linings in brain) as shown in Fig. 5, panel 2, c and f (also see below). No Large T staining was observed in tissues from control noninjected mice (not shown). In order to confirm that IEM cells chimerized endothelial components rather than macrophages or other cell types, double-labeling studies were performed using anti-Large T and anti-vWFVIII antibodies to simultaneously label IEM derived cells and endothelial cells,

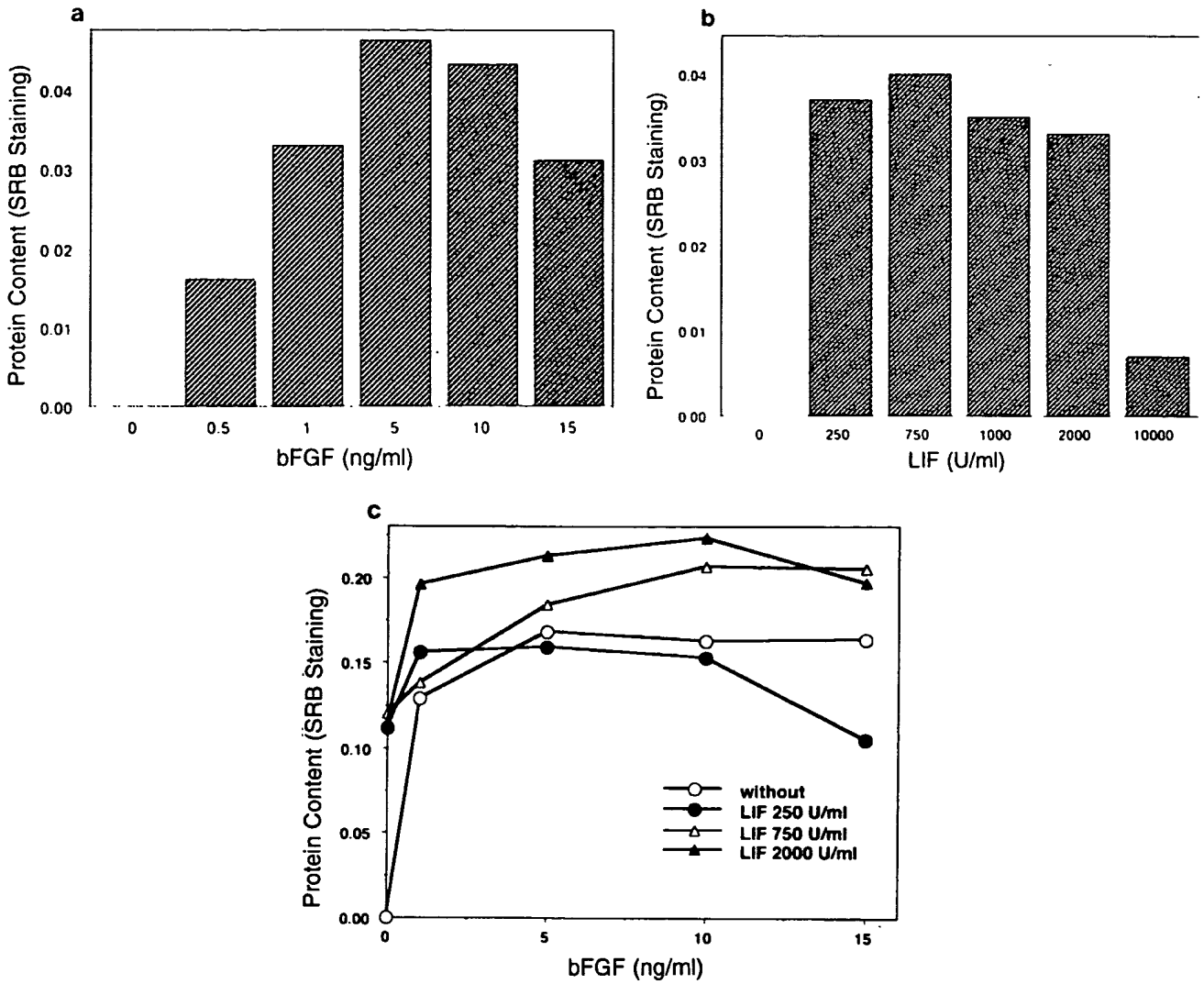


FIG. 3. Growth of IEM cells in response to bFGF and LIF after 24 (a,b) and 72 hr (c) culture in cytokines as assessed by SRB staining of total protein (ordinate) versus the concentrations of either bFGF (ng/ml), LIF (U/ml), or combinations of bFGF and LIF (abscissa). Values are expressed as increases over control (no added cytokine) from means of pentuplicates from four separate experiments; standard errors (not shown) were below 0.01 for each point.

respectively. The Large T and vWFVIII epitopes were co-localized at clusters of cells most heavily concentrated in red pulp areas (those regions containing the endothelial sinusoids) as shown at high-dry magnification (Fig. 5, panel 3, a-c). A double-stained IEM-derived cell photographed under oil immersion is shown in Fig. 5, panel 3, d-f. These results confirm endothelial chimerization by IEM cells in spleen. Unfortunately, chimerism of the thymus could not be analyzed immunocytochemically due to limiting quantities of this tissue. The Large T immunostaining pattern was both nuclear and cytoplasmic. Although T antigen distribution in cells expressing SV40 is mainly nuclear, some T antigen can also be detected immunocytochemically in the cytoplasm and at the cell surface, as described previously in SV40 Large T transgenic mice (Cheah *et al.*, 1995). For this reason, the T antigen staining pattern appears somewhat heterogeneous, since sections of positive cells contain variable portions of cytoplasm and nucleus, depending on the plane of section. No Large T immunostaining was observed in those organs showing no genomic IEM chimerism such as lung and uterus (not shown). Animals showing IEM contribution were housed under standard conditions into adulthood. Neither tumors nor ill health were observed in either the IEM chimeric pups at birth or in the adult IEM chimeric mice at ages up to 5 months. No significant alteration in Large T protein could be detected by Western blot in cytokine exposed versus control cultures (not shown). In addition, since it was readily detected in the IEM chimeras, Large T does not appear to be suppressed by either cytokine exposure or host influence in a long-term manner. These results demonstrate

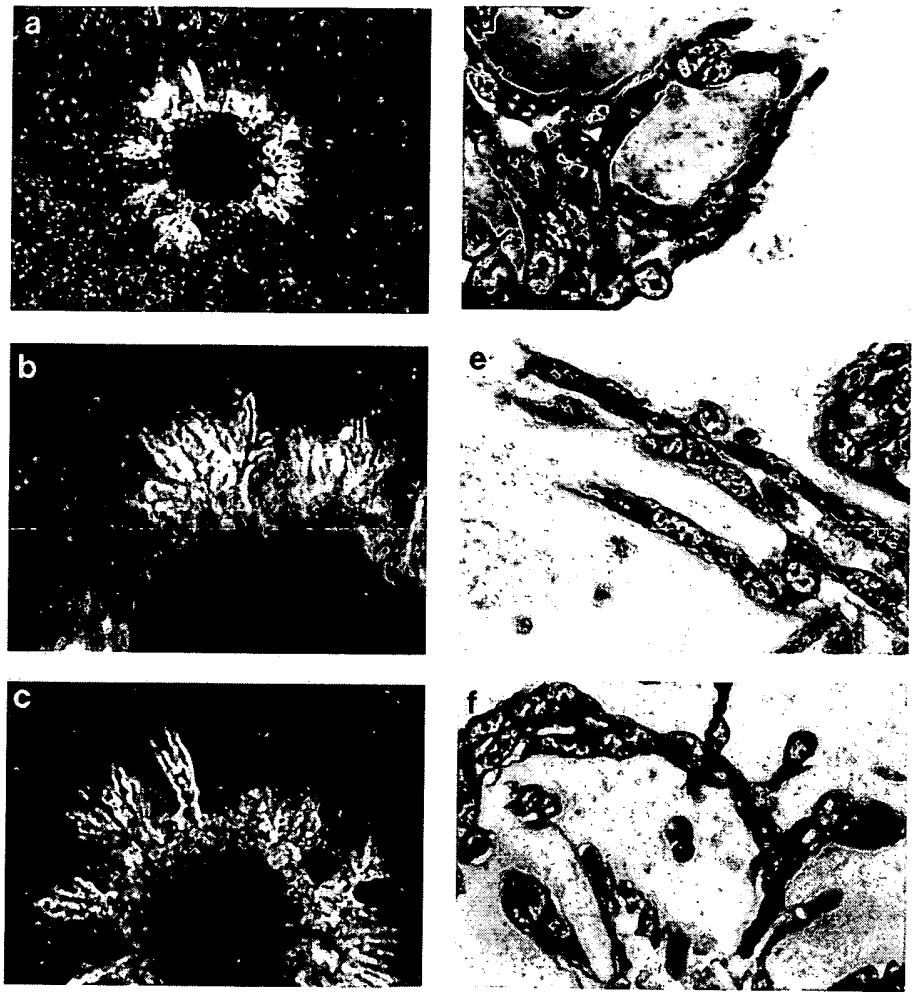


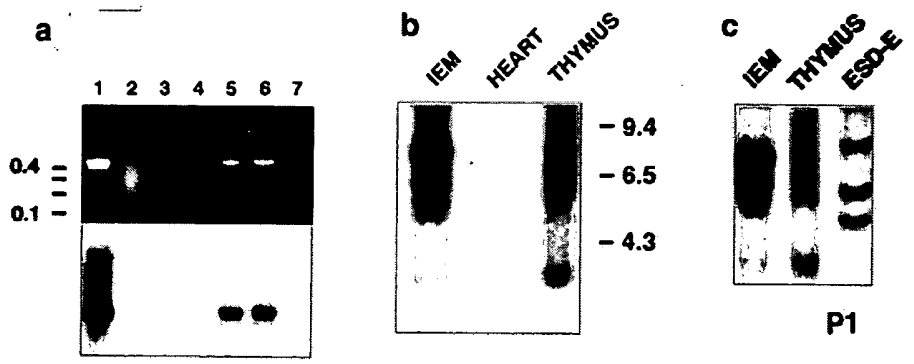
FIG. 4. Examples of blood vessel-like structures projecting from cytokine-induced IEM colonies 3 days after transfer to Matrigel; phase-contrast (a) 10×; (b,c) 20×. (d–f) Thin sections of the same vasculogenic IEM Matrigel colonies as displayed above, showing cross-sectional (d) and longitudinal (e) histological views of sprouting vessels composed of walls consisting of single endothelial cell thickness. Microcapillary-like anastomoses are also observed and are shown in (f).

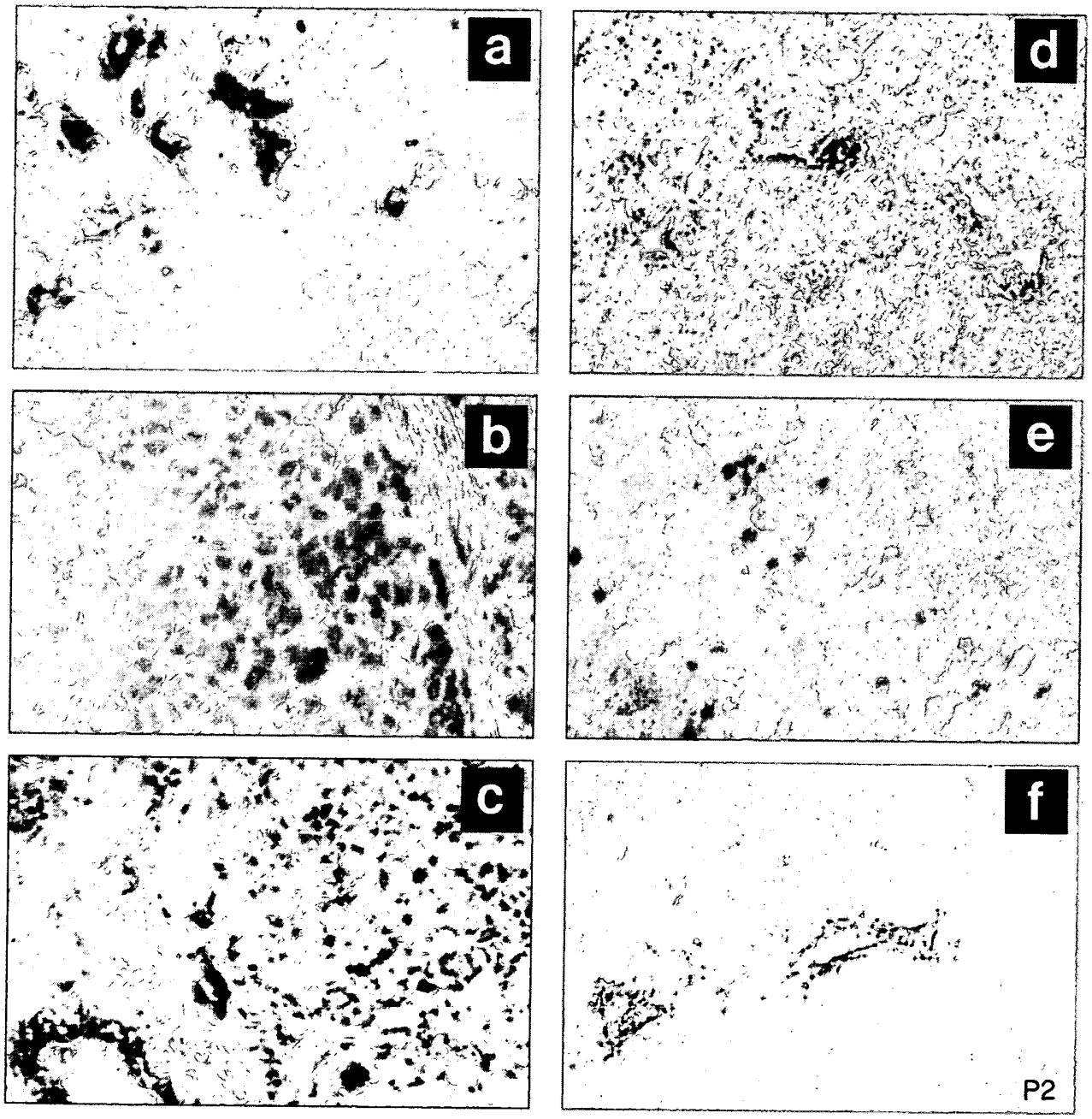
that cytokine stimulation of IEM cells leads to the induction of a specific differentiative pathway permitting IEM cells to develop *in vivo* with endogenous endothelium.

DISCUSSION

The IEM cell line, derived from differentiated ES cells, expresses vWFVIII, VCAM-1, and PECAM-1 (CD31). The IEM cells also internalize aLDL *in situ*. These characteristics together clearly define the IEM cell line as having an endothelial phenotype (Wagner *et al.*, 1982; Osborn *et al.*, 1989; Baldwin *et al.*, 1994; Voyta *et al.*, 1984; Alles and Bosslet, 1988). Each of these markers (vWFVIII, VCAM-1, and PECAM-1) are expressed at embryonic stages of endothelial development, with PECAM-1 being one of the earliest adhesion molecule markers for developing endothelial cells in the embryo (Baldwin *et al.*, 1994; Vecchi *et al.*, 1994). In addition, we have recent evidence that IEM cells express the *flk-1* RTK protein and that *flk-1* is regulated upon cytokine exposure of IEM (manuscript in preparation). *Flk-1* is expressed in preangioblast mesoderm cells and angioblast progenitors and is required for normal vascular and hematopoietic development (Shalaby *et al.*, 1995). The IEM cells contribute to blastocyst chimeras *in vivo* only after exposure to bFGF/LIF. However, vasculogenic colonies derived from cytokine-stimulated IEM cells *in vitro* are complex in structure but homogenous in cellularity, with no evidence of accompanying spontaneous hematopoiesis. These results indicate that the IEM cell line probably represents an early endothelial subset, most likely immortalized at a point between the time after differentiation from a bipotential hemangioblast and the point of early maturation into vessel-competent endothelium.

The response of IEM cells to bFGF and LIF exposure involves proliferation, a shift in morphology to a tightly





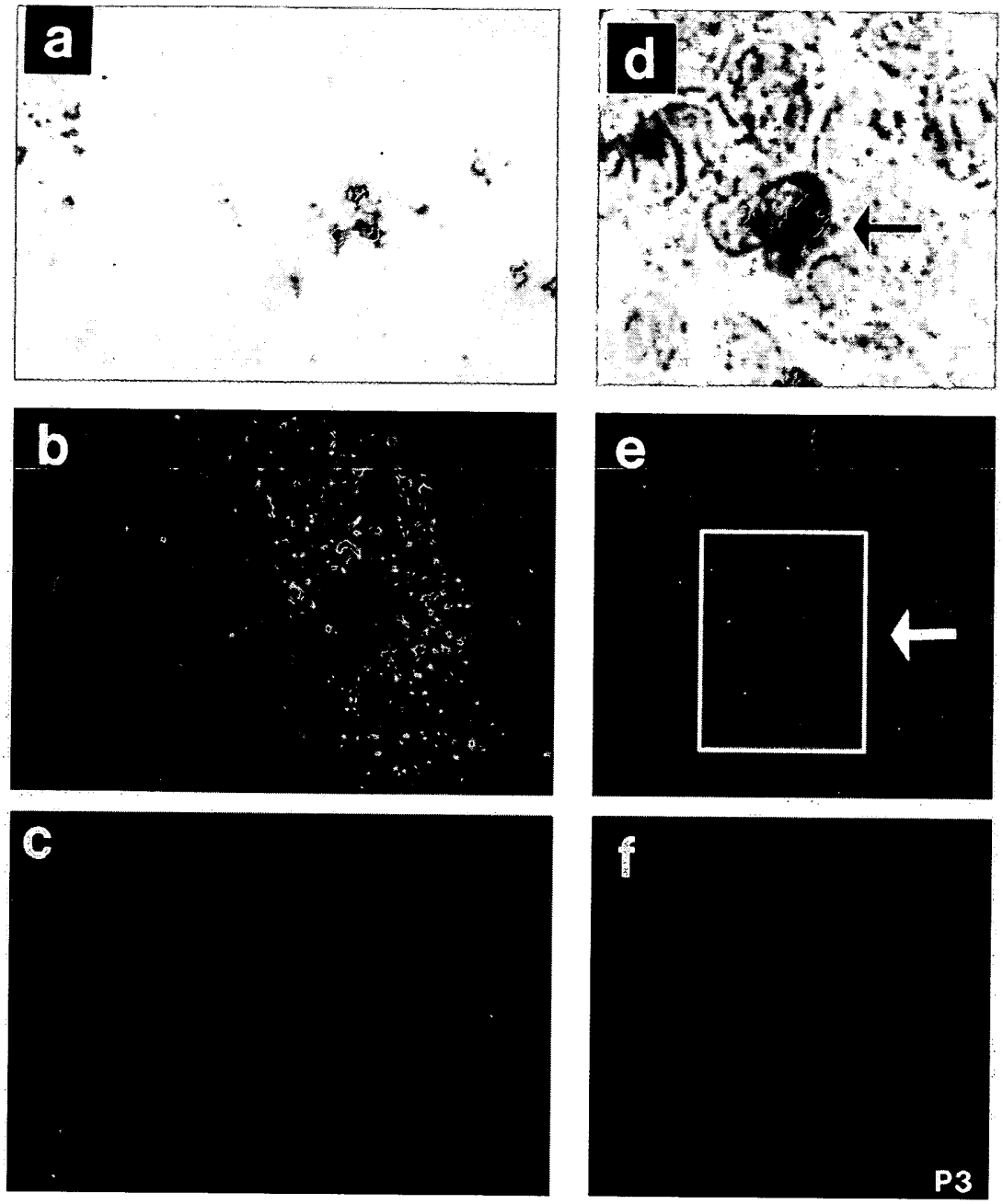


FIG. 5. Cytokine exposure permits IEM cells to chimerize microvascular endothelium after blastocyst injection. Panel 1: (a) Representative PCR amplification of SV40 Large T sequences in tails of offspring from blastocysts injected with cytokine-exposed IEM cells (top). Corresponding Southern hybridization of PCR gel using an SV40 Large T specific cDNA probe (bottom). IEM, lane 1; negative offspring, lanes 2, 3, 4, and 7; positive offspring, lanes 5 and 6. (b) Representative genomic Southern blot analysis of the thymic tissue from a male IEM chimera. Genomic DNA was digested with *BclI*. The blotted DNA was then probed with a 1500-bp Large T fragment specific for Large T sequences upstream of the single *BclI* restriction site in the vector. (c) Genomic Southern blot analysis of IEM, lane 1, chimeric thymic tissue, lane 2, and a separate, simultaneously derived SV40 immortalized cell line ESD-2, lane 3; Southern blotting was carried out as described for b. Panel 2: Immunocytochemistry demonstrating IEM-derived Large T positive cells in IEM chimeric tissues. (a) Spleen from an IEM chimera showing Large T positive cells localized in the red pulp sinusoids surrounding trebeculae. (b) Adjacent section incubated with isotype control antibody showing no staining. (c) vWFVIII staining of a section closely adjacent to those shown in (a and b) demonstrating the location of endothelial cells in red pulp sinusoids surrounding trebecular areas. (d) Brain from an IEM chimera showing Large T positive cells localized in microcapillary endothelium. (e) Adjacent section incubated with isotype control antibody showing no staining. (f) vWFVIII staining of a section adjacent to those shown in (d and e) demonstrating the location of endothelial cells in brain microvascular capillaries. Panel 3: Double label immunocytochemistry showing identical fields of a cluster of vWFVIII-SV40 large T positive IEM-derived cells localized in a splenic red pulp region (a,b) and high-power view of an individual IEM-derived cell expressing both endothelial vWFVIII and SV40 Large T antigen in the splenic red pulp of an IEM chimera (d,e). (a,d) Immunoperoxidase staining of SV40 T (red stain). (b,e) Simultaneous fluorescence staining of the same field with anti-vWFVIII (green granular staining). (c,f) Negative control spleen sections incubated with normal rabbit serum in place of anti-vWFVIII followed by FITC-tagged anti-rabbit (background staining). a,b, and c were photographed at 40×, while d,e, and f were photographed under oil immersion at 100X.

TABLE 1
Incidence of IEM Chimerism for Blastocyst Injections with either Cytokine-Exposed or Untreated IEM Cells

Treatment of cells	Injected embryos transferred	Individuals pregnant	Number of pups born	Number chimeric
βFGF + LIF	45	6/6	21	6
None	51	5/6	15	0

packed colony phenotype, and the aquisition of the ability to form complex vasculogenic structures *in vitro* after transfer to matrix layers. Moreover, bFGF/LIF stimulation permits IEM cells to codevelop with normal endothelial progenitors in blastocyst chimeras *in vivo*. The cytokine requirements in IEM cells suggest a role for bFGF/LIF synergy in vasculogenic processes. Although such a response has not been demonstrated previously for endothelial cells, transdifferentiative changes in response to combinations of bFGF and LIF have been described for cells in other systems. For example, primordial germ cells and developing skeletal muscle cells undergo profound growth and phenotypic shifts in response to combinations of bFGF and LIF or bFGF and CNTF (another closely related cytokine of the LIF family), respectively (Resnick *et al.*, 1992; Matsui *et al.*, 1992; English and Schwartz, 1995). The IEM response demonstrates that bFGF/LIF induces a specific activation pathway directing IEM cells to form vascular structures and suggests that the bFGF/LIF combination may have similar effects on intrinsic endothelial projenitors *in vivo*. Alternatively, bFGF/LIF may not be important *in vivo* since untreated IEM cells do not chimerize blastocysts. However, it is likely that endogenous bFGF/LIF *in vivo* may exert an effect upon endothelial cells or endothelial precursors at a stage much later than that represented by 3.5-day blastocysts, which have not yet developed primitive mesoderm, the precursor to angioblasts. Moreover, 3.5-day blastocysts have not yet developed extraembryonic endodermal tissues, which are thought to induce angioblasts to commence vasculogenesis and have also been shown to be a major endogenous source of both bFGF and LIF in the developing embryo (Christ *et al.*, 1991; Wilms *et al.*, 1991; Yasuda *et al.*, 1992; Rathjen *et al.*, 1990). Survival of IEM cells in the blastocoele cavity after injection may be dependent upon mechanical requirements related to adhesion or migration. The injected IEM cells may require exposure to exogenous bFGF/LIF to fulfill such requirements until endogenous cytokines produced to act upon developing endothelium are present. Since chimerism is not found in animals resulting from blastocyst injections of untreated IEM cells, these IEM cells must have died and/or become diluted out at some point after injection, supporting the argument that exogenous bFGF/LIF is required for their support. In addition, the full cytokine-induced shift to colony phenotype occurs over a period of 3 days in culture. IEM cells introduced into blastocysts without bFGF/LIF preexposure may not have sufficient time of stimulation by endogenous cytokines in the blastocyst for these cells to shift phenotype in synchrony with developmental events directing endogenous endothelial cell differentiation. That IEM cells show reduced and delayed vasculogenic colony formation compared to cytokine exposed IEM *in vitro* also lends support to the requirement and specificity for a timely bFGF/LIF stimulus. If the cytokine-induced IEM response was not involved with a developmental mechanism associated with endothelial differentiation, one of three possible experimental outcomes could be predicted to occur after blastocyst injection. The first possibility is that IEM contribution would not have been observed in vascular compartments of animals resulting from blastocyst injection with cytokine-treated cells. In the second possible outcome, IEM contribution would have been observed in random nonvascular compartments of animals resulting from blastocyst injection with either untreated or cytokine-treated cells. The third possibility is that both untreated and cytokine-exposed IEM cells would have proliferated uncontrolled as tumors in the developing blastocysts and disrupted embryo development, as is observed with embryonal carcinoma cells (Rossant and McBurney, 1982). That none of these outcomes occurred and that specific vascular chimerism was detected only after cytokine exposure indicates that the combined bFGF/LIF stimulus must activate a novel vasculogenic response pathway in the IEM cells. These results suggest a similar mechanism may play a role in activating endogenous vasculogenesis in the developing embryo.

Both bFGF and LIF, in addition to their respective receptor components, are expressed in the developing embryo or yolk sac during the stages in which vasculogenesis occurs, making plausible an argument for their combined role in vasculogenesis during development (Rathjen *et al.*, 1990; Saito *et al.*, 1992; Yasuda *et al.*, 1992). In the avian embryo, bFGF is expressed at stages during which angioblasts arise and appears to potently induce the expression of VEGF receptor during vasculogenesis (Mitrani *et al.*, 1990; Flamme *et al.*, 1995). In mouse, it has been demonstrated that endodermally derived bFGF acts upon the endothelial progenitors expressing bFGF receptor (flg) (Yasuda *et al.*, 1992). As null mutants have not been described for these genes it is difficult to predict the absolute requirement for bFGF/flg during mouse vasculogenesis *in vivo*. However, in quail blastodisc isolate cultures, it is clear that vasculogenesis absolutely depends upon the presence of bFGF (Krah *et al.*, 1994). Vasculogenesis occurs spontaneously in mouse embryoid bodies, but the presence and level of endogenous bFGF may be quite different in each species as may be a requirement for a timed burst of this cytokine to stimulate endothelial precursors (Risau *et al.*, 1988; Wang *et al.*, 1992). Although the phenotype of the LIF null mouse does not show any vascular abnormalities (Stewart *et al.*, 1992), other redundant cytokines such as CNTF or IL-6, which signal through the same receptor component (gp130; Ip *et al.*, 1992), may compensate for any effects that absent LIF family members may exert on developing endothelium. Mice harboring a

null mutation for the low-affinity LIF receptor (LIFRβ) show abnormalities in placental vasculature, suggesting that LIF effects signaled through this particular receptor subset may indeed be important for the development of subgroups of endothelial cells (Ware *et al.*, 1995). Other cytokines and RTK receptors including VEGF, *flk-1*, *flt-1*, *tek*, *Tie-1*, and *Tie-2* have been demonstrated by either expression studies and/or gene targeting to be important for various aspects of the development of the vascular tree (Millauer *et al.*, 1993; Fong *et al.*, 1995; Yamaguchi *et al.*, 1993; Sato *et al.*, 1993; Dumont *et al.*, 1994; Sato *et al.*, 1993). Whether or not these other cytokines might interact with bFGF/LIF *in vivo* remains to be investigated. However, we have recently observed that the IEM cells also express the VEGF RTK flk-1 protein, suggesting that IEM cells might also be capable of responding to VEGF (manuscript in preparation). Since the vasculogenesis process in quail appears to involve interactions between bFGF and the VEGF receptor *flk-1* (Flamme *et al.*, 1995), the IEM model will be a useful means for further investigating the details of signaling mechanisms associated with cytokine interactions during vasculogenesis in a homogenous mouse system.

The response of the IEM cells to bFGF and LIF involves both proliferation and a transdifferentiative shift to a different phenotype. Growth response of IEM cells indicates that a specific range of concentrations of bFGF and LIF, either alone or in combination, are optimal in inducing growth (5–10 ng/ml and 250–2000 U/ml, respectively) of the IEM cells. The specific range of concentrations of bFGF and LIF inducing maximal growth are the same when used alone or in combination. The combinatory growth effect of bFGF and LIF is partially additive, suggesting a saturation of the growth stimulation response. At higher concentrations of bFGF and LIF (10–15 ng/ml and 2000–10,000 U/ml range, respectively), suboptimal growth stimulation is achieved. Similar growth responses have been observed in other systems. Decreased effects at higher cytokine concentrations have been previously described for primordial germ cells (Resnick *et al.*, 1992; Matsui *et al.*, 1992). LIF has recently been shown to inhibit angiogenesis, although the concentrations of LIF required to achieve such inhibition are an order of magnitude higher than concentrations normally effective for stimulating growth in the IEM cells (Pepper *et al.*, 1995) or for stimulating signal transduction in a number of other well-defined LIF-responsive cell types including myeloid leukemia cells, neurons, and primordial germ cells (Lord *et al.*, 1991; Ip *et al.*, 1992; Resnick *et al.*, 1992; Matsui *et al.*, 1992). LIF concentrations reported to be inhibitory to angiogenesis fall within the refractory region of the IEM response (10,000 U/ml), suggesting that endothelial responses to LIF may be highly concentration dependent. Moreover, LIF appears to have highly variable effects upon different endothelial cell subsets, with major differences observed in growth inhibition of aortic versus microvessel endothelium (Ferrara *et al.*, 1992). Our observations coupled with others in the literature suggest that endothelial responses to LIF vary considerably with the heterogeneity of endothelial cells from different organs and different stages of differentiation. These observations also emphasize the importance of deriving separate model systems mimicking the heterogeniety of endothelial cell subsets during both development and adulthood.

There is little understanding of the mechanisms by which combinations of cytokines and growth factors influence cellular processes. Signaling pathways of individual cytokines and growth factors are not fully understood. Therefore, it is of central importance to elucidate the mechanism by which bFGF and LIF induce the reprogramming of IEM cells into a vasculogenic phenotype. Our preliminary results show that the immediate-early responses of IEM to bFGF and LIF stimulation are similar to those observed in other systems (unpublished results). The molecular events determining the developmental IEM response to these cytokines (phenotypic shift) probably results from subsequent downstream events such as transcriptional changes rather than a unique immediate-early response pattern. We are currently investigating the transcriptional responses to bFGF and LIF in the IEM system.

The effect of bFGF/LIF exposure in IEM cells involves proliferation and differentiation simultaneously. The only other case in which a similar response pattern has been described for these cytokines *in vitro* is in primordial germ cells, where bFGF and LIF, in the presence of stem cell factor (SCF), induces transdifferentiation and proliferation simultaneously, leading to the proliferation of primordial germ cells as embryonic stem cells (Resnick *et al.*, 1992; Matsui *et al.*, 1992). Interestingly, HUVEC cells, which should approximate normal mature endothelium, failed to show a similar phenotypic shift in response to bFGF/LIF exposure, suggesting that this response is specific for early embryonic stages of endothelial differentiation. Our results provide the first evidence that vasculogenesis may also involve simultaneous proliferative and differentiative changes leading to a profound shift in phenoype.

The IEM cells display cytokine responsiveness, undergo vasculogenesis *in vitro* and can chimerize developing and adult vascular structures despite the expression of SV40 Large T antigen. We have been unable to detect any significant changes in the level of expression of SV40 protein during the *in vitro* responses of IEM cells to cytokine stimulation, indicating that such phenotypic changes are not accompanied by changes in expression of Large T in the IEM cells. Therefore, the bFGF/LIF-mediated proliferation, colony formation, and vasculogenesis are most likely not associated with changes in the expression level of SV40 Large T. We have been unable to suppress Large T protein expression using specific antisense oligonucleotides for the purpose of addressing whether the differentiative or growth responses are directly affected by SV40 expression (unpublished observations). However, since SV40 Large T antigen can be expressed in normally differentiating and proliferating tissues from embryonic stages through adulthood without any appreciable adverse effects (Shida *et al.*, 1992), it is likely that the expression of SV40 Large T antigen by the IEM cells does not affect the proliferative and differentiative changes observed upon cytokine exposure.

Various vascular endothelial cell lines have been previously described, some of which were isolated from polyoma virus middle T induced embryonic vasoformative tumors (Obeso *et al.*, 1990; Dubois *et al.*, 1991). Cell lines from HUVEC, which represent relatively more differentiated endothelium, have also been isolated after spontaneous (Cockerill *et al.*, 1994) or SV40 Large T antigen immortalization (Vicart *et al.*, 1993). Primary cultures of HUVEC have also been used, although these cultures tend to be highly heterogeneous from one preparation to the next (Watson *et al.*, 1995). Since IEM cells were derived from a developmentally early source (embryonic stem cells), they model endothelium at a considerably earlier stage than previously described systems. The vasculogenic behavior of the IEM cells differs from capillary-like structures inducible on Matrigel or collagen in other systems described previously (Obeso *et al.*, 1990; Cockerill *et al.*, 1994). First, the vasculogenic potential of IEM cells is induced by bFGF/LIF rather than exposure to basement membrane components alone. Second, the external and internal structures of IEM-derived vasculogenic colonies are more complex than the more homogenous tubes described in other systems (Obeso *et al.*, 1990; Cockerill *et al.*, 1994). Vessel-forming colonies derived from IEM cells contain newly sprouting vessels, variably sized extending vessels with open lumens, and anastomosing cavities representing developing capillary extensions. These IEM-derived colonies more closely resemble the developing embryonic vasculature in which rapid proliferation is accompanied by simultaneous differentiation, sprouting, and elongation of angioblasts.

The IEM cells chimerize the vascular component of a limited group of organs. This is interesting in that the IEM cells, although derived from pluripotential ES cells, are committed to the endothelial lineage, as evidenced by the range of endothelial markers expressed by these cells. We are not aware of any other differentiated cell types which are capable of tissue specific chimerization in the embryo. The ability of the IEM cells to chimerize a limited range of organs may be due to the stage of differentiation at which they were immortalized. Alternatively, our data also raise the intriguing possibility that embryonic endothelial cells may possess a considerable degree of phenotypic heterogeneity at early developmental stages. Perhaps local factors in the seperately developing organs influence committed endothelial cells differently. The isolation and analysis of additional organ-specific embryonic endothelial cell lines will help to address this issue. Finally, the cytokine-induced changes modeled by the IEM system may not be limited to embryogenesis. Perhaps the combined action of bFGF and LIF may also influence the plasticity of vascular endothelium during adult physiological processes such as neoplasia and wound healing.

ACKNOWLEDGMENTS

We thank Dr. Stuart Orkin of Childrens Hospital Boston for his kind assistance with chimera construction. We also thank M. Brown for the pZIPTEX packaging cell line and plasmid; Ginnette M.-Le and Ramana Tantravahi for karyotype analysis; Saori Kashiki, Sandha Rao, Carol Moore, and Lisa Adams for technical help; Dr. Sue Heffelfinger for providing primary cultures of HUVEC cells as well as an extensive and helpful critique of the manuscript. We also thank Genetics Institute for generously providing LIF. R.L.G. was supported by a postdoctoral fellowship from the MRC of Canada. This work was supported by NIH grant HD27322 to R.J.A.

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Received for publication November 17, 1995
Accepted May 1, 1996